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Evidence for a phosphorylation-induced conformational change in phospholamban from the effects of three proteases

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The effect of the proteases trypsin, thermolysin and papain on the cardiac membrane protein phospholamban was examined before or after phosphorylating the protein with the catalytic subunit of cyclic AMP-dependent protein kinase. The sensitivity of phospholamban to digestion by trypsin and thermolysin was greatly reduced by phosphorylation, suggesting that phospholamban undergoes a conformational change upon phosphorylation. It is suggested that this change in conformation is the mechanism by which phospholamban phosphorylation relieves its inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase pump.

Phospholamban; Sarcoplasmic reticulum; cyclic AMP; Phosphorylation; Ca^{2+}-ATPase; (Cardiac muscle)

1. INTRODUCTION

Phospholamban is a membrane protein intrinsic to cardiac sarcoplasmic reticulum (SR). When it is phosphorylated by cyclic AMP-dependent protein kinase, the activity of the SR Ca^{2+} pump ATPase is stimulated by an increase in its affinity for Ca^{2+} [1,2].

Electrophoretic analysis of purified phospholamban has led to the conclusion that the protein exists as a pentamer of identical subunits, each with an M_r of approx. 5500 [3,4]. Recently, the N-terminal sequence of phospholamban has been determined by two groups [4,5]; one of these sequences seems to represent the structure from residue 10 onwards [5]. Based on these data, and the results of experiments which examined the effects of proteases on the detergent-solubilised, purified protein [6], a low-resolution model of the tertiary and quaternary structure has been produced [5,6]. Two essential features of this model are as follows. Firstly, phospholamban has a hydrophobic, membrane-anchoring domain which is linked to a cytoplasmic, protease-sensitive domain containing the phosphorylation sites of the protein. Secondly, phospholamban exists as an ordered 'channel' structure held together by hydrophobic interactions in the hydrophobic domain. The validity of this model to the structure of phospholamban when imbedded in the SR membrane has still to be determined.

This paper describes the results of protease action on membrane-bound phospholamban, and confirms that in the SR phospholamban subunits are held together by a protease-resistant domain. However, in contrast to the detergent-solubilised protein, the accessibility of proteases to the hydrophilic domain depends on whether or not phospholamban is phosphorylated. A preliminary report of these findings has been published previously [7].

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2. MATERIALS AND METHODS

SR vesicles were prepared by an adaptation of the method in [8]. All operations were performed at 4°C. Fresh guinea pig hearts were washed in 0.9% (w/v) NaCl and then homogenized using a Polytron PT10 in 0.29 M sucrose, 5 mM NaN_3, 30 mM Tris-HCl, 0.5 mM EDTA, 15 mM 2-mercaptoethanol, 50 μM phenylmethylsulphonyl chloride, pH 8, at 4°C. The homogenate was centrifuged at $5000 \times g$ for 10 min and then at $10000 \times g$ for 10 min. The resulting supernatant was filtered through glass wool and centrifuged at $225000 \times g$ for 30 min. The pellet was resuspended using a glass/teflon hand homogenizer in 15 ml of 0.6 M KCl, 2 mM EGTA, 3 mM $MgCl_3$, 0.58 M sucrose, 15 mM Hepes-NaOH, pH 7.6, to solubilise remaining myofibrils. After incubation at 4°C for 30 min the membranes were recentrifuged at $225000 \times g$ for 30 min, resuspended in 1 ml of 25 mM Hepes-NaOH, pH 7, frozen in aliquots in liquid nitrogen and stored at −70°C.

The catalytic (C) subunit of cyclic AMP-dependent protein kinase was purified to homogeneity by the method of Reimann and Beham [9] and stored at −70°C in 50% (v/v) glycerol, 100 mM sodium phosphate, 1 mM EGTA, pH 7. The specific activity of the kinase towards histone III-S was 0.4 μmol/min per mg C-subunit protein.

Membranes were either phosphorylated with C-subunit, then digested with proteases and subjected to electrophoresis on SDS-polyacrylamide gels, or else digested first, then phosphorylated and subjected to electrophoresis. After each incubation, SR was rapidly centrifuged to a pellet at $350000 \times g$ for 15 min at 4°C in the TLA-100.2 rotor of a Beckman TL-100 tabletop ultracentrifuge. SR was phosphorylated by incubation at 1–2 mg protein/ml with 100 mM Hepes-Tris, 25 mM $MgCl_2$, 20 mM EGTA, 1 mM dithiothreitol, pH 7, and 2 μg/ml C-subunit at 30°C. After 10 min preincubation, [γ-^{32}P]ATP (spec. act. 50 TBq/mol) was added to 60 μM and after a further 10 min the reaction was stopped by the addition of excess EDTA. SR was digested by incubation in 25 mM Hepes-NaOH, pH 7, at 30°C with trypsin, thermolysin or papain at the concentrations and for the times given in section 3. Incubations with thermolysin also contained 0.1 mM $CaCl_2$. Digestion was stopped by cooling to 4°C, and by adding phenylmethanesulphonyl chloride to 200 μM in the case of trypsin, or EGTA to 1 mM in the case of thermolysin. Proteases were obtained from Worthington (trypsin) or Sigma (thermolysin and papain).

15% SDS-polyacrylamide gels were run according to [10]. SR pellets were solubilised by incubation at 30°C in 2% (w/v) SDS, 20% (w/v) glycerol, 125 mM Tris-HCl, 40 mM dithiothreitol for 1 h with frequent agitation. Myosin, cytochrome *c* (Sigma) and aprotinin (Sigma) were used as M_r standards. Autoradiographs were exposed within the linear range of absorbance [11], and were scanned using a Bio-Rad model 620 video densitometer.

3. RESULTS AND DISCUSSION

When SR was incubated with the C-subunit of cyclic AMP-dependent protein kinase, phosphorylation of one protein of M_r 29000 accounted for >90% of the protein phosphorylation in the membrane vesicles (fig.1, tracks 1). When subjected to SDS-polyacrylamide gel electrophoresis, phospholamban migrates in a characteristic way as either a 'high' or 'low' M_r form [12,13]. If samples are solubilised in SDS at low temperatures (e.g. 30°C), then phospholamban migrates as a multimer with an apparent M_r of 22000–29000. However, if samples are solubilised in SDS at 100°C then a monomeric form (with an apparent M_r of ~5500) is observed. In these experiments heating samples for 4 min at 100°C caused the dissociation of the protein into subunits of apparent M_r 5500 (fig.1a) confirming that the observed phosphorylated protein was phospholamban. Incubation of the membranes for 1 h at 30°C did not cause marked dephosphorylation of phospholamban (fig.1b, track 1).

The susceptibility of phospholamban to digestion by trypsin has been a matter of controversy, with the phosphorylated protein reported to be either protease-sensitive [4,6,14,15,16], or resistant to trypsin attack [1,17,18]. Fig.1b shows that phosphorylated phospholamban is resistant to digestion by trypsin at concentrations up to 0.1 mg/ml for 1 h. Subjecting membranes to 1 μg/ml trypsin for 15 min virtually eliminated Coomassie blue staining of the gel (not shown) and phosphorylation of minor bands (fig.1b, track 2), in-

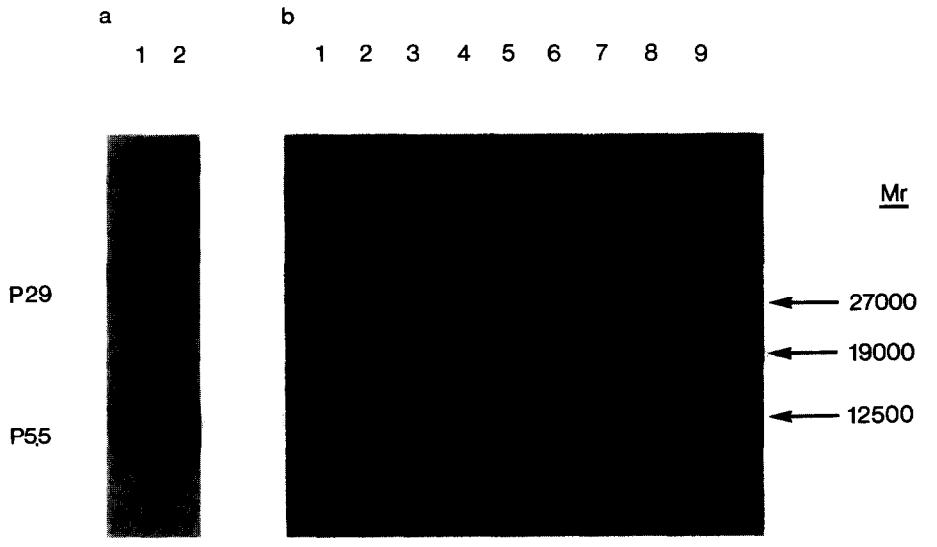

Fig.1. Autoradiographs showing the phosphorylation of SR proteins by cyclic AMP dependent protein kinase. (a) SR was phosphorylated and subjected to electrophoresis as described in section 2 (track 1), or additionally heated in SDS sample buffer at 100°C for 4 min prior to electrophoresis (track 2). P29 and P5.5 are the phospholamban forms of apparent M_r 29000 and 5500, respectively. (b) Effect of trypsin on phosphorylated phospholamban. Prephosphorylated membranes were incubated with trypsin at the following concentrations and times: 0 μg/ml (track 1), 1 μg/ml (track 2, 15 min incubation; track 3, 60 min), 10 μg/ml (track 4, 5 min; track 5, 15 min; track 6, 60 min) or 100 μg/ml (track 7, 5 min; track 8, 15 min; track 9, 60 min). Digested membranes were centrifuged and then run onto SDS gels. The arrows show the migration of M_r standards.

dicating that phospholamban was selectively resistant to trypsin compared to other proteins. At high trypsin concentrations there was evidence of a slight reduction of the apparent M_r of phospholamban, the clipped form having an apparent M_r of 27000 (fig.1b, tracks 7-9). This change in apparent M_r is unlikely to be the result of dephosphorylation [6], because in control incubations (without trypsin or at lower concentrations) a reduction in apparent M_r was not observed (fig.1b, tracks 1-6).

Unphosphorylated SR was incubated either in buffer alone or with trypsin for 30 min at 30°C at concentrations ranging from 10 to 500 μg protease/ml. At all concentrations of trypsin tested at least 95% of phospholamban was digested, as shown by the subsequent loss of a radioactive band on SDS gels after incubation with C-subunit and [γ-^{32}P]ATP (table 1). It would therefore appear that in intact SR, trypsin only causes major digestion of unphosphorylated phospholamban, a result not observed with the purified protein [6]. In all cases where phosphorylated phospholamban has been found to be protease-sensitive, the protein has either been denatured [14] or detergent-solubilised [4,6,15,16], and this suggests that some element of the native conformation of phospholamban in SR is responsible for the protection of the protein to protease attack.

An alternative explanation for the above results is that in the unphosphorylated protein, trypsin cut the Arg-Arg or Arg-Ala bonds of the phosphorylation site [6], preventing subsequent recognition (and hence phosphoryaltion) by the C-subunit. Such an action of trypsin on the phosphorylated protein would leave the phosphate attached to the residual protein, and the clipping effect observed in fig.1b might be taken as evidence that this mechanism is occurring in the above experiments. Thermolysin, by contrast, would not be expected to cleave within the phosphorylation site, and so if this mechanism were correct, thermolysin should digest phosphorylated phospholamban. Phosphorylated SR was incubated with 1 mg thermolysin/ml for various times as shown in table 1. Within 5 min of incubation most Coomassie blue staining

Table 1

Effect of phosphorylation by cyclic AMP-dependent protein kinase on the proteolysis of membrane-bound phospholamban

Time of digestion (min)	Phosphorylation before digestion			Digestion before phosphorylation		
	Trypsin	Thermo-lysin	Papain	Trypsin	Thermo-lysin	Papain
0	100	100	100	100	100	100
5	77	112	–	–	81	–
10	–	104	–	–	34	<5
15	76	–	9	–	33	–
30	–	–	–	4	–	–
40	–	107	–	–	9	–
60	82	82	1	–	5	<5

Values are the percentage of phosphorylated phospholamban remaining in the high-M_r form (compared to control without digestion) after various incubation periods with one of three proteases either before or after incubation with the C-subunit of cyclic AMP-dependent protein kinase. Thermolysin was used at 1 mg/ml and papain at 0.5 mg/ml. Trypsin was used at 50 μg/ml in the incubation before phosphorylation and 100 μg/ml in the incubation after phosphorylation

material on SDS gels had disappeared (not shown), indicating the digestion of most protein, and the phospholamban form of apparent M_r 29 000 had been clipped to a band of apparent M_r 27 000. However, there was no significant loss of phosphate from phospholamban after 1 h (table 1). By contrast, when SR was first incubated with thermolysin for various times, the phospholamban available for subsequent phosphorylation by cyclic AMP-dependent protein kinase was reduced in a graded manner (fig.2, table 1). As with trypsin, this ability of phosphorylation to protect against proteolysis was not observed with purified, detergent-extracted phospholamban [6]. Because phosphorylated phospholamban is resistant to thermolysin as well as trypsin, and because this effect is only observed in native membranes, it is concluded that phosphorylation by cyclic AMP-dependent protein kinase induces a conformation change in phospholamban which renders the sites of digestion to trypsin and thermolysin inaccessible to these two enzymes.

A different result was obtained using papain as the proteolytic enzyme. In this case, phospholamban was rapidly digested, whether prephosphorylated or not, by 0.5 mg papain/ml (table 1). It would therefore seem that the site of papain digestion on phospholamban is exposed regardless of whether phospholamban is in the phosphorylated or unphosphorylated conformation. However, this effect was observed in the presence or absence of

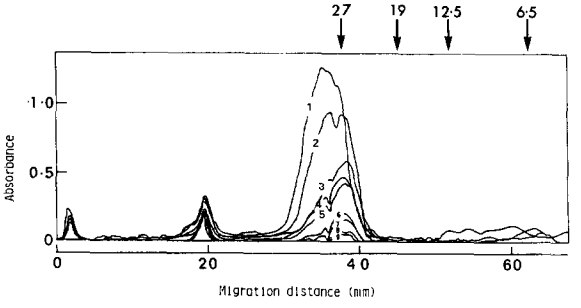

Fig.2. Video densitometer scans of autoradiographs of thermolysin-digested SR after phosphorylation and SDS-polyacrylamide gel electrophoresis. SR was first digested with 1 mg/ml thermolysin at 30°C for 0, 5, 10, 15, 20, 40, 60, 90 or 120 min (lines 1–9, respectively) and then phosphorylated with cyclic AMP-dependent protein kinase (see section 2). The arrows indicate the apparent M_r values ($\times 10^{-3}$) of standard proteins. The phosphorylated band at approx. 19 mm comigrated with the C-subunit of cyclic AMP-dependent protein kinase.

1 mM dithiothreitol, indicating that papain may have been acting in a non-specific manner.

In no case in this series of experiments did proteolysis cause dissociation of phospholamban pentamers into monomers. This is in agreement with the model of [5] that the protease-resistant, hydrophobic domains of the molecule hold the subunits together. If this were not the case, then it would be expected that as phospholamban was digested a stepwise reduction in apparent M_r would occur as subunits dissociated from the multimer. The digestion with thermolysin (fig.2) shows that this is not the case. However, these experiments reveal an additional complexity in the 3-D structure of phospholamban, in that the protein is able to adopt different conformations depending on its phosphorylation state. Inui et al. [19] have shown that unphosphorylated phospholamban inhibits the SR Ca^{2+}-ATPase and that the function of phosphorylation is to relieve this inhibition. Presumably, then, phosphorylation causes a conformational change in phospholamban which reduces its affinity for a binding site on the Ca^{2+}-ATPase. This work provides clear evidence for such a conformational change. In addition, Kirchberger et al. [18] have very recently shown a correlation between the digestion of unphosphorylated SR phospholamban by trypsin with an activation of ATP-dependent Ca^{2+} uptake by the vesicles. Taken together with the results presented here, this strongly suggests that a phosphorylation-induced conformational change in phospholamban is responsible for relieving its inhibition of the Ca^{2+}-ATPase.

We are presently in the process of characterizing a panel of monoclonal antibodies to phospholamban [20]. Preliminary results with some of these antibodies suggest that they interact with particular conformational states of phospholamban, providing a means of probing phospholamban conformation during phosphorylation in native SR vesicles.

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